Remineralization of Enamel Subsurface Lesions by Sugar-free Chewing Gum Containing Casein Phosphopeptide-Amorphous Calcium Phosphate

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ABSTRACT

Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) exhibit anticariogenic potential in laboratory, animal, and human *in situ* experiments. The aim of this study was to determine the ability of CPP-ACP in sugar-free chewing gum to remineralize enamel subsurface lesions in a human *in situ* model. Thirty subjects in randomized, cross-over, double-blind studies wore removable palatal appliances with six human-enamel half-slabs inset containing subsurface demineralized lesions. The appliances were inserted immediately before gum-chewing for 20 min and then retained for another 20 min. This was performed four times *per* day for 14 days. At the completion of each treatment, the enamel half-slabs were paired with their respective demineralized control half-slabs, embedded, sectioned, and subjected to microradiography and densitometric image analysis, for measurement of the level of remineralization. The addition of CPP-ACP to either sorbitol- or xylitol-based gum resulted in a dose-related increase in enamel remineralization, with 0.19, 10.0, 18.8, and 56.4 mg of CPP-ACP producing an increase in enamel remineralization of 9, 63, 102, and 152%, respectively, relative to the control gum, independent of gum weight or type.

KEY WORDS: enamel remineralization, sugar-free chewing gum, casein phosphopeptide-amorphous calcium phosphate.

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INTRODUCTION

Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) have been demonstrated to have anticariogenic potential in laboratory, animal, and human *in situ* experiments (Reynolds *et al.*, 1995, 1999; Reynolds, 1987, 1997, 1998, 1999; Rose, 2000). Casein phosphopeptides (CPP) containing the cluster sequence –Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu- have a remarkable ability to stabilize amorphous calcium phosphate (ACP) in metastable solution. Through the multiple phosphoseryl residues, the CPP binds to forming nanoclusters of ACP, preventing their growth to the critical size required for nucleation and phase transformation (Reynolds, 1998).

In a human *in situ* enamel demineralization study, a 1.0% w/v CPP-ACP solution used twice daily produced a 51 ± 19% reduction in enamel mineral loss caused by frequent sugar-solution exposure (Reynolds, 1998). The twice-daily use of the 1.0% CPP-ACP solution resulted in a 144% increase in calcium level and a 160% increase in inorganic phosphate level in the inter-enamel plaque recovered from the removable intra-oral appliance (Reynolds, 1998). These results suggested an anticariogenic mechanism for the CPP-ACP where the CPP stabilize and localize ACP at the tooth surface, thereby buffering plaque pH, depressing enamel demineralization, and enhancing remineralization. Recent *in vitro* experiments (Reynolds, 1997) have shown that CPP-ACP solutions promote remineralization of enamel subsurface lesions. In these experiments, a 1.0% w/v CPP-ACP solution produced 63.9 ± 20.1% remineralization of enamel subsurface lesions over a 10-day period.

Caries clinical trials of sugar-free chewing gum have shown that the gum is non-cariogenic and, in fact, can have an anticariogenic effect through the stimulation of saliva (Möller and Poulsen, 1973; Glass, 1983; Kandelman and Gagnon, 1990; Mäkinen *et al.*, 1995; Beiswanger *et al.*, 1998). Sugar-free gum, therefore, may be an excellent delivery vehicle for a safe and effective additive capable of promoting enamel remineralization. In this paper, we demonstrate the ability of CPP-ACP (Recaldent™) in sugar-free gum to remineralize enamel subsurface lesions in a human *in situ* model.

MATERIALS & METHODS

Subject Recruitment

Three randomized, double-blind, crossover studies were conducted (Table 1). Approval for the studies was obtained from the University of Melbourne Human Research Ethics Committee, and all subjects provided informed consent. Healthy adult subjects were recruited for the three studies from among the staff and post-graduate students of the School of Dental Science, the University of Melbourne (Table 2). An intra-oral examination confirmed that each had at least 22 natural teeth with no current caries activity, periodontal disease, or other oral pathology. None of the subjects was using antibiotics or medications which could have affected salivary flow. We measured unstimulated salivary flow rates by instructing the subjects to lean forward with their heads tilted downward, allowing saliva to flow into a pre-weighed centrifuge tube for exactly 2 min. We measured salivary flow rates stimulated by gum-chewing by instructing the subjects to

chew on sugar-free gum (Warner Lambert Consumer Group, Pfizer Inc., Morris Plains, NJ, USA) for exactly 2 min while allowing all saliva produced to flow into a pre-weighed centrifuge tube. The volume of saliva collected in the two-minute period was determined from the final weight of the centrifuge tube plus saliva.

Intra-oral Appliances

Removable mid-palatal acrylic appliances were fabricated to cover the palate from the first premolars (or canines in some cases) to the last tooth in the arch (usually 3rd molars). The base was held in place by 4 narrow-gauge stainless steel circumferential clasps. The acrylic base was relieved 10 mm from the palatal surfaces of the posterior teeth to leave an area clear for the gum bolus during chewing. Anterior, posterior, and lateral dams were created in the acrylic base to improve retention and also to prevent the gum from passing underneath the base of the appliance. Two bilateral troughs (15 mm long, 7 mm wide, and 2 mm deep) parallel to the posterior teeth were cut into the acrylic base about 3 mm medial to the lateral margins of the appliance. These troughs were prepared to house 3 demineralized enamel half-slabs per trough by means of wax retention.

Preparation of Enamel Subsurface Lesions

Occlusal and gingival mesiodistal windows (1 x 7 mm^2) separated from each other by 1 mm were prepared on 8 x 4 mm^2 surfaces of human third molar enamel slabs as described previously (Reynolds, 1997). Subsurface lesions were created in the enamel windows by means of the Carbopol method of White (1987) as modified by Reynolds (1997). After demineralization, each enamel slab was sectioned through the midline of each window into two half-slabs with 4 x 4 mm^2 surfaces, and the cut surface of each half-slab was covered with nail varnish. One half-slab of each pair was retained as the demineralization control and stored in a humidified environment. The other enamel half-slab of the pair was inset into the intra-oral appliance for the remineralization protocol. Six enamel half-slabs were inset into each appliance, 3 on each side. The enamel half-slabs were removed after every treatment period, and the test and control enamel half-slabs were paired, then embedded, sectioned, and analyzed.

Study Protocols

The protocols of the three *in situ* studies were identical except for the specific polyol (sorbitol or xylitol), weight, and type (slab or pellet) of sugar-free gum, CPP-ACP dose, and number of treatments (Table 1). The sugar-free chewing gums were prepared by the Adams Division of Warner Lambert, Pfizer Inc., with CPP-ACP (Recaldent™) obtained from Bonlac Foods Limited (Melbourne, Australia). The gums were provided as coded products and were stored at room temperature. All chewing-gum treatments were double-blinded and randomized. The randomization schedule and product code were retained by the Clinical and Consumer Test Supplies Department of the Warner Lambert Consumer Healthcare Group of Pfizer Inc. and were released only after completion of each clinical study. For each study, all subjects crossed over to each randomly assigned treatment, with at least one week between treatments.

Table 1. Gum Type, Weight, and CPP-ACP Dose *per* Treatment for Each Study

Study	Gum Type	Sugar Alcohol	Gum Weight *per* Treatment	Treatments 1	2	3	4
				Sugar-free Gum CPP-ACP (mg)			
CPP-ACP dose response/sorbitol-based pellet gum	Pellet	Sorbitol	3.0 g[a]	No[b]	0[c]	18.8	-[d]
CPP-ACP dose response/sorbitol-based slab gum	Slab	Sorbitol	1.9 g[a]	No	0.19	18.8	56.4
CPP-ACP dose response/xylitol-based pellet gum	Pellet	Xylitol	3.0 g[a]	No	0	10.0	18.8

[a] For the pellet-gum studies, 2 pieces of pellet gum (1.5 g *per* piece) were used, and for the slab-gum study, 1 piece (1.9 g) was used for each chewing period.
[b] No treatment (no gum chewing).
[c] Dose of CPP-ACP (mg) in total weight of chewing gum used *per* chewing period.
[d] Not done.

All subjects used standard fluoride dentifrice for the duration of each study and chewed the gums at their natural chewing frequency for 20 min 4 times daily for 14 days. Subjects chewed the gum at the following times: 10:00 a.m., 11:30 a.m., 2:00 p.m., and 3:30 p.m. The appliances were worn for the 20 min of gum-chewing and for 20 min following. In the case of the no-treatment control, the appliances were worn for 40 min. At all other times, the appliances were stored in sealed moist plastic bags at room temperature. Subjects were instructed not to eat or drink while wearing the appliance and to rinse and clean their appliances using a fluoride-free denture cleanser paste and toothbrush provided. They were informed not to brush the area containing the enamel blocks. No alterations were made to the subjects' diet and oral hygiene procedures for the duration of each study. After completion of each treatment period, the enamel blocks were removed from the appliances for processing.

Enamel Sectioning and Microradiography

At the completion of each treatment period, each remineralization enamel half-slab, paired with its demineralization control half-slab, was dehydrated in absolute alcohol. The pair of half-slabs was embedded

Table 2. Subject Characteristics and Salivary Flow Rates

Study	Subject Age (yrs)	No. of Subjects	Male/ Female	Unstimulated Salivary Flow Rate (mL/min)	Stimulated Salivary Flow Rate (mL/min)[a]
CPP-ACP dose response/sorbitol-based pellet gum	33 ± 7	10	6M/4F	0.7 ± 0.4	3.0 ± 1.0[b]
CPP-ACP dose response/sorbitol-based slab gum	30 ± 7	10	7M/3F	0.4 ± 0.2	3.1 ± 1.5[b]
CPP-ACP dose response/xylitol-based pellet gum	34 ± 6	10	6M/4F	0.7 ± 0.5	3.3 ± 0.5[b]

[a] Stimulated salivary flow rates were measured while subjects chewed sugar-free gum for 2 min.
[b] Significantly higher ($p < 0.001$ ANOVA/Tukey) than unstimulated value.

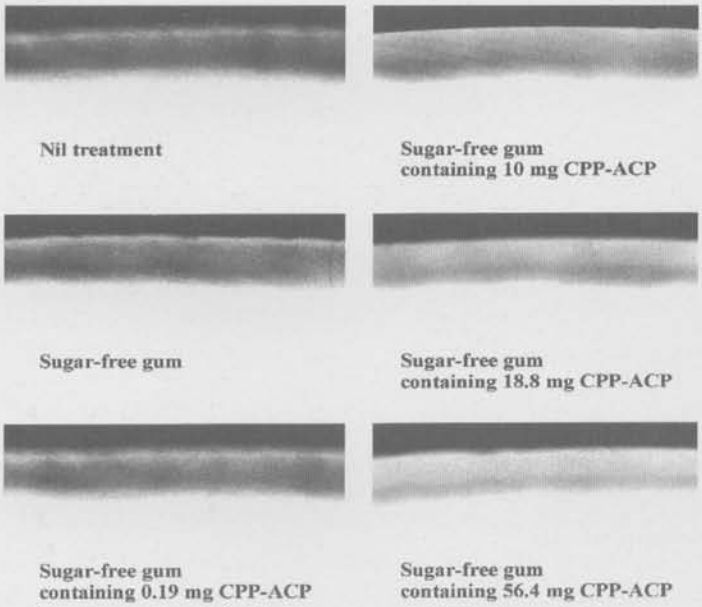

Figure. Representative microradiographic images of the enamel subsurface lesions showing the effect of each CPP-ACP dose and the no-treatment control.

and sectioned through the midline of both half-lesions as described previously (Reynolds, 1997). The sections were then lapped down to 80 ± 5 μm by means of a RotoPol-21/RotoForce 4 lapping instrument (Struers, Rødovre, Denmark) with 1200- and 2400-grit lapping paper. Each section, which contained the remineralized half-lesion and the paired demineralized control half-lesion from the same enamel slab, was radiographed together with an aluminum stepwedge as described previously (Reynolds, 1997), by means of Microchrome High Resolution glass plates (Type 1A, Microchrome, San Jose, CA, USA).

Microdensitometric Image Analysis

Radiographic images of the lesions were viewed through a Dialux 20 microscope (Ernst Leitz, Wetzlar, Germany), and the images were acquired by means of a video camera (Sony DXC 930P). The images were digitized by a frame-grabber (Imaging Frame Grabber, Scion Corporation, Frederick, MD, USA) under the control of imaging software (Optimate version 4.2). Images of the lesions and the neighboring areas of sound enamel were scanned by means of the program's line luminance function that gives readings in grey values. An area free of artifacts or cracks was selected for analysis, and 6 scans were performed and averaged to provide a representative scan of each lesion. Each scan was comprised of 200 readings taken from the tooth surface through the lesion to sound enamel. The aluminum stepwedge image on each slide was also scanned. We used linear regression to convert each grey value into the equivalent thickness of aluminum. The enamel section thickness was measured and vol% mineral data were determined by the equation of Angmar *et al.* (1963). The image of the median strip between the 2 lesions was scanned 6 times and averaged to give a control sound-enamel densitometric profile. Similarly, we scanned the lesion images (remineralization windows and demineralization control windows) on the gingival and occlusal sides of the median strip, as close as possible to the median strip but avoiding any irregularities commonly found at the lesion edges, and determined the vol% mineral profiles.

Data Analysis

The vol% mineral profile of each enamel block's demineralized and remineralized lesion was compared with the median sound enamel vol% mineral profile of the same section (Reynolds, 1997). The percentage enamel remineralization [%R = (1 - ΔZr/ΔZd) x 100] was determined by means of trapezoidal integration as described previously, where ΔZd represents the difference between the area under the sound enamel profile and the demineralized enamel profile, and ΔZr represents the difference between the area under the sound enamel profile and the remineralized enamel profile (Reynolds, 1997). Data (%R for each treatment) were statistically analyzed by a one-way classification analysis of variance (ANOVA) with the Tukey *a posteriori* multiple comparison (Sokal and Rohlf, 1969).

RESULTS

The chewing of sugar-free gum produced a four- to seven-fold increase in salivary flow rate over the two-minute period in all subjects (Table 2). Preliminary studies indicated that no CPP-ACP could be detected in the gum bolus after 10 min of chewing; hence, for the *in situ* studies, subjects were instructed to chew the test gum for 20 min and then to retain the intra-oral appliance for a further 20-minute period. The 660 demineralized subsurface lesions used for the *in situ* studies were consistent in depth (110 ± 9 μm) and in volume of mineral lost, with a mean ΔZd of 3222 ± 545 vol% min.μm. A clear dose-response relationship was obtained for the CPP-ACP level in the sugar-free gum and enamel subsurface remineralization (Table 3, Fig.). No significant difference was observed between the sorbitol-based and xylitol-based gums in their ability to stimulate saliva (Table 2) or

Table 3. Remineralization of Enamel Subsurface Lesions (%R)

Treatment[a]	CPP-ACP Dose Response/ Sorbitol-based Pellet Gum	CPP-ACP Dose Response/ Sorbitol-based Slab Gum	CPP-ACP Dose Response/ Xylitol-based Pellet Gum	Average	Increment (Δ%R)[e]	
No treatment	3.3 ± 1.0[b]	4.3 ± 1.6[c]	3.2 ± 1.0[d]	3.6 ± 0.9[g]	N.D.	
3.0 g gum not containing CPP-ACP	9.1 ± 1.2[b]	N.D.	8.9 ± 1.1[d]	9.0 ± 0.4[h]	N.D.	
0.19 mg CPP-ACP in 1.9 g gum	N.D.	9.8 ± 1.8[c]	N.D.	9.8 ± 1.8[h]	0.8[f]	(9)
10.0 mg CPP-ACP in 3.0 g gum	N.D.	N.D.	14.7 ± 0.9[d]	14.7 ± 0.9[i]	5.7	(63)
18.8 mg CPP-ACP in 1.9 g or 3.0 g gum	18.4 ± 3.0[b]	17.1 ± 2.5[c]	19.1 ± 1.6[d]	18.2 ± 1.7[j]	9.2	(102)
56.4 mg CPP-ACP in 1.9 g gum	N.D.	22.7 ± 3.4[c]	N.D.	22.7 ± 3.4[k]	13.7	(152)

[a] Gum chewed 4 times daily for each study. For the pellet-gum studies, 2 pieces of pellet gum (1.5 g each) were used, and for the slab-gum study, one piece (1.9 g) was used for each chewing period.
[b,c,d] Significantly different ($p < 0.001$, ANOVA/Tukey) from all other values in column.
[e] Increment in %R relative to the control sugar-free gum not containing CPP-ACP.
[f] Percentage increase remineralization relative to the control sugar-free gum not containing CPP-ACP.
[g,h,i,j,k,l] Significantly different ($p < 0.001$, ANOVA/Tukey) from all other values in column not similarly marked.
N.D.= Not done.

remineralize subsurface enamel lesions (Table 3). The addition of CPP-ACP to either the sorbitol- or xylitol-based gums at 10.0 mg, 18.8 mg, or 56.4 mg produced a significant ($p < 0.001$) increase in enamel remineralization, with a 63%, 102%, and 152% average increase, respectively, relative to the sugar-free gum not containing CPP-ACP (Table 3). No significant correlation was found between individual unstimulated or stimulated salivary flow rates and percentage enamel remineralisation values obtained for any of the treatments.

DISCUSSION

The results of the 3 *in situ* studies showed that the addition of CPP-ACP to sugar-free chewing gum significantly enhanced remineralization of enamel subsurface lesions in a dose-related manner, independent of gum weight or type (Fig., Table 3). The no-treatment (no gum-chewing) involved subjects' wearing the intra-oral appliance for 40-minute periods 4 times a day for 14 days (total exposure of 37.3 hr), and this resulted in 3.6 ± 0.9% enamel remineralization. Although the placement of the appliance into the mouth may have transiently stimulated salivary flow, the majority of this remineralization would have resulted from unstimulated saliva. Unstimulated saliva is supersaturated with respect to tooth mineral, and the contact of the enamel slabs in the appliance with a film of unstimulated saliva would have resulted in this small but significant remineralization. The chewing of either the sorbitol or xylitol sugar-free gum for 4 20-minute periods a day for 2 wks resulted in 9.0 ± 0.4% enamel remineralization, which represents over 2 times the level of remineralization found with the no-treatment control. Chewing gum provided a masticatory and gustatory stimulus to salivation whereby salivary flow in the subjects increased four- to seven-fold over a two-minute period (Table 2). Dawes and MacPherson (1991) have shown that salivary flow rate is increased during gum chewing to a peak of about 10 times the unstimulated flow rate during the first min of gum chewing, followed by a decrease to a level of 3 times the unstimulated flow rate after 20 min. Hence, this substantial increase in salivary flow rate over the 20-minute chewing period, together with the higher calcium and phosphate ionic concentrations in stimulated saliva (Lagerlöf and Oliveby, 1994), is consistent with the observed increase in enamel remineralization. The remineralization of enamel subsurface lesions by 3.6 ± 0.9% during the no-treatment period represented remineralization by unstimulated saliva for a total exposure period of 37.3 hr. However, the 9.0 ± 0.4% remineralization obtained with the sugar-free gum represented remineralization from the same total exposure period of 37.3 hr but included 18.7 hr of stimulated saliva from gum chewing.

In a similar *in situ* model, Creanor *et al.* (1992) compared the effects of a sorbitol chewing gum with a no-gum control on enamel remineralization. In this study, enamel sections containing subsurface lesions were positioned lingual to the mandibular molars by means of a removable appliance. Subjects wore the appliances for a seven-week period except during oral hygiene procedures. Subjects chewed 5 sticks of gum *per* day, each for 20 min, immediately after meals and snacks. The sorbitol-gum treatment resulted in 18.2% enamel remineralization compared with 12.1% remineralization for the no-gum control. This difference was not significantly different ($p > 0.05$), due to the large variances, which could be attributed to a long total exposure time (1176 hr) relative to the gum chewing time (87.5 hr) and the variable effects of meals and snacks. In the current study, appliances were worn only when subjects were chewing gum and for a 20-minute period immediately after chewing, thus minimizing the effects of variables unrelated to gum chewing.

Leach *et al.* (1989) also compared enamel remineralization by a sorbitol gum with a no-gum control over 21 days in an *in situ* model. Cast silver bands cemented to lower first molars contained demineralized enamel blocks which were covered with gauze. The depth and $\triangle Zd$ values of the subsurface lesions were smaller than those of the lesions used in the current study. Gum was chewed after meals and snacks for 20 min 5 times a day. Remineralization values were 16.8% for the no-gum control and 28.9% for the sorbitol gum treatment. The higher level of enamel remineralization (28.9%) effected by the sorbitol gum in the Leach *et al.* (1989) study, compared with that obtained with the equivalent gum in the present study (9.0 ± 0.4%), can be attributed to the smaller lesions used and the much greater chewing time (35 hr) and total exposure time (504 hr) relative to 18.7 hr and 37.3 hr, respectively, for the current study.

The levels of remineralization obtained in the present study with the sugar-free gum containing 18.8 mg CPP-ACP and 56.4 mg CPP-ACP were 18.2 ± 1.7% and 22.7 ± 3.4%, respectively. The 18.8 mg CPP-ACP produced a 102% increase, and the 56.4 mg CPP-ACP produced a 152% increase in enamel remineralization relative to the sorbitol gum not containing CPP-ACP (Table 3). These results indicate that the remineralization potential of sugar-free gum is significantly enhanced by the inclusion of CPP-ACP. In fact, the level of remineralization obtained with the 56.4 mg CPP-ACP gum in 18.7 hr of chewing and a total exposure of 37.3 hr was comparable with that obtained with normal sugar-free gum in the Leach *et al.* (1989) study with smaller lesions and 35 hr of gum chewing and a total salivary exposure of 504 hr.

The addition of CPP-ACP to either the sorbitol- or xylitol-based sugar-free gum resulted in a CPP-ACP dose-related increase in enamel subsurface remineralization (Table 3). Combining all the data from the 3 *in situ* studies (Table 3) clearly demonstrates a dose response, with 0.19 mg, 10.0 mg, 18.8 mg, and 56.4 mg of CPP-ACP in sorbitol/xylitol sugar-free gum producing an increase in enamel remineralization of 9%, 63%, 102%, and 152%, respectively, relative to the control gum, independent of gum weight or type (Table 3). The increased enamel remineralization found in this study by inclusion of CPP-ACP in sugar-free chewing gum is consistent with previous studies showing the anti-cariogenic and remineralization potential of CPP-ACP in solution (Reynolds *et al.*, 1995; Reynolds, 1997) and the proposed mechanism of the localization of amorphous calcium phosphate at the tooth surface by the CPP depressing enamel demineralization and enhancing remineralization (Reynolds, 1998).

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